Isolation of a *Microsporum canis* Gene Family Encoding Three Subtilisin-Like Proteases Expressed *in vivo*

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Microsporum canis* is the main agent of dermatophytosis in dogs and cats and is responsible for frequent zoonosis. The pathogenesis of the disease remains largely unknown, however. Among potential fungal virulence factors are secreted keratinolytic proteases, whose molecular characterization would be an important step towards the understanding of dermatophytic infection pathogenesis. *M. canis* secretes a 31.5 kDa keratinolytic subtilisin-like protease as the major component in a culture medium containing cat keratin as the sole nitrogen source. Using a probe corresponding to a gene's internal fragment, which was obtained by polymerase chain reaction, the entire gene encoding this protease named *SUB3* was cloned from a *M. canis* λEMBL3 genomic library. Two closely related genes, termed *SUB1* and *SUB2*, were also cloned from the library using as a probe the gene coding for *Aspergillus fumigatus* 33 kDa alkaline protease (ALP). Deduced amino acid sequence analysis revealed that SUB1, SUB2, and SUB3 are secreted proteases and show large regions of identity between themselves and with subtilisin-like proteases of other filamentous fungi. Interestingly, mRNA of *SUB1*, *SUB2*, and *SUB3* were detected by reverse transcriptase nested-polymerase chain reaction from hair of experimentally infected guinea pigs. These results show that *SUB1*, *SUB2*, and *SUB3* encode a family of subtilisin-like proteases and strongly suggest that these proteases are produced by *M. canis* during the invasion of keratinized structures. This is the first report describing the isolation of a gene family encoding potential virulence-related factors in dermatophytes. *Key words: dermatophyte/keratinase/subtilisin-like protease. J Invest Dermatol 119:830–835, 2002

Pathogenic dermatophytes are parasitic fungi that share the ability to invade keratinized structures such as hair, nails, and stratum corneum, causing superficial infections called dermatophytosis in both humans and animals (Weitzman and Summerbell, 1995). *Microsporum canis* is the main agent of dermatophytosis in dogs and cats (Scott *et al*, 2001) but is also a frequent zoonotic agent, as shown by the increasing prevalence of human infections in many European countries (Lunder and Lunder, 1992). Human infection occurs mainly by direct contact with infected cats (De Vroey, 1985), which are considered as the natural hosts and the reservoir for *M. canis* (Scott *et al*, 2001). The fact that cats can be asymptomatically infected (Mignon and Losson, 1997) enhances the risk for both human and animal contamination as such animals are responsible for occult and massive dissemination of fungal material into their environment (Symoens *et al*, 1989).

Among potential virulence factors of dermatophytes, secreted proteases, and especially keratinolytic ones, have been investigated the most. They could provide the fungus with nutrients, by degrading keratin into easily assimilable metabolites (Apodaca and McKerrow, 1989a), and allow the invasion of keratinized structures (Apodaca and McKerrow, 1989b). Furthermore, keratinolytic proteases could be involved in the control of host defense mechanisms (Grappel and Blank, 1972; Collins *et al*, 1973). Keratinolytic proteases have been isolated from different species of dermatophytes including *Trichophyton rubrum* (Meevootisom and Niederpruem, 1979; Asahi *et al*, 1985; Lambkin *et al*, 1996), *Trichophyton mentagrophytes* (Yu *et al*, 1968; 1971; Tsuboi *et al*, 1989), and *M. canis* (Takiuchi *et al*, 1982; 1984; Lee *et al*, 1987; Mignon *et al*, 1998a; Brouta *et al*, 2001). Despite the presumed role of these keratinases in pathogenesis, very few studies have dealt with their *in vivo* expression. Interestingly, a well-characterized 31.5 kDa keratinolytic subtilisin-like serine protease was previously shown to be secreted by *M. canis* as the major component, *in vitro*, in a minimal medium enriched with cat keratin (Mignon *et al*, 1998a). Moreover, the *in vivo* expression of this keratinase was demonstrated in hair of both *M. canis* naturally infected cats (Mignon *et al*, 1998b) and experimentally infected guinea pigs (Mignon *et al*, 1999a), suggesting its role in the pathogenesis of this dermatophytic infection. The role of this protease, thought to be an essential *M. canis* virulence factor, should be further investigated, however.

No dermatophyte keratinolytic protease has been characterized so far at the molecular level. Such a characterization would therefore be an important step towards the understanding of dermatophytic infection pathogenesis. This paper describes the isolation and characterization of a *M. canis* gene family encoding

Manuscript received October 22, 2001; revised January 11, 2002; accepted for publication February 5, 2002

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Abbreviations: *SUB*, subtilisin-like protease (gene); SUB, subtilisin-like protease (protein).

three subtilisin-like proteases (SUBs) including the 31.5 kDa keratinase previously isolated by Mignon *et al* (1998a). Focusing on the potential role of these three SUBs in the pathogenesis of *M. canis* dermatophytosis, the *in vivo* transcription of their genes was demonstrated in hair of experimentally infected guinea pigs.

MATERIALS AND METHODS

Experimental infection of guinea pigs Two specific pathogen-free 3-mo-old female guinea pigs of the Hartley strain (B & K Universal, Humberside, U.K.) were cutaneously infected with *M. canis* essentially as described by Van Cutsem (1989). Briefly, inoculum consisting of mycelia and spores collected from 13-d-old slopes cultures on Sabouraud medium and suspended in a honey:water (1:2 vol/vol) mixture was applied on a 15 cm^2 back skin surface previously clipped and scarified. Negative controls consisted of two guinea pigs exposed to the same procedure except that the honey:water mixture did not contain any fungus. Animal infection was regularly monitored using clinical, Wood's light, and microscopic examinations. Animal experiments were approved by the local ethic committee (University of Liège).

Strains, plasmids, and growth conditions *M. canis* strain IHEM 15221 (Brussels, Belgium) was used for the construction of the genomic library, the *in vitro* isolation of fungal RNA, and guinea pig infection. The fungus was grown for 7 d in liquid Sabouraud medium and for 12 d in a minimal liquid medium containing cat keratin as the sole nitrogen source (Mignon *et al*, 1998a) for DNA and RNA extraction, respectively. *Escherichia coli* LE392 was used for the propagation of bacteriophage λEMBL3 (Promega, Madison, WI). All plasmid subcloning experiments were performed in *E. coli* DH5α (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) or *E. coli* TOP10 (Invitrogen, Carlsbad, CA) using plasmids pMTL21 (Chambers *et al*, 1988) and pPicZαB (Invitrogen) or pCR®4Blunt-TOPO® (Invitrogen), respectively.

***M. canis* genomic library and gene cloning** Genomic DNA was isolated as described by Girardin and Latgé (1994) and purified using genomic-tips (Qiagen, Hilden, Germany). The isolated DNA was partially digested by *Sau* 3AI. DNA fragments ranging from 10 to 20 kb were purified from low melting agarose (Bio-Rad, Richmond, CA) and inserted into bacteriophages using the λEMBL3 *Bam* HI arm cloning system (Promega). Approximately 3×10^4 recombinant plaques of the genomic library were immobilized on nylon membranes (Genescreen, NEN® Life Science Products, Boston, MA). The membranes were prehybridized at 42°C for 45 min in hybridization solution [5 × standard sodium citrate, 20% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate, and 100 μg per ml of denatured salmon sperm DNA (Boehringer, Mannheim, Germany)]. Thereafter, the filters were probed at 42°C for 13 h under low-stringency conditions with two different ^{32}P-labeled probes used independently. The first probe consisted of the gene of the 33 kDa alkaline protease (ALP) of *Aspergillus fumigatus* (Jaton-Ogay *et al*, 1992), obtained by polymerase chain reaction (PCR) amplification using primers 5′-CGGGATCCGCCTTGACC-ACTCAAAAGGG-3′ and 5′-GCAGATCTTTAAGCATTGCCATTG-TAGGCAAG-3′ and a 2.2 kb *Sph* I fragment as template DNA (Jaton-Ogay *et al*, 1992). The second probe consisted of a DNA fragment obtained by PCR amplification using *M. canis* genomic DNA as template and degenerated sense primer 5′-ACIACICAICCIAA(C/T)GCICCIACITGGGG-3′ and antisense primer 5′-TGIGGIG(A/T)IGCCATIGAIGTICC-3′. These primers were based on the N-terminal amino acid sequence of the mature protease (Mignon *et al*, 1998a) and on the highly conserved amino acid sequence region around the serine of the catalytic triad of the serine proteinases of the subtilisin family (S8) (Rawlings and Barrett, 1994). The ^{32}P-labeling of probes was performed with [α-^{32}P]-dATP (Amersham Pharmacia Biotech) using a random-primed DNA labeling kit (Boehringer). The membranes were then exposed to X-ray film after two 15 min washes at 40°C in washing solution (2 × standard sodium citrate, 1% sodium dodecyl sulfate). Positive plaques were purified and the associated bacteriophage DNAs were isolated as described by Grossberger (1987). Agarose gel electrophoresis of restricted recombinant bacteriophage λEMBL3 DNA and southern blotting were performed using standard procedures (Sambrook *et al*, 1989). Hybridizing fragments were subcloned into pMTL21 according to standard protocols (Sambrook *et al*, 1989). Sequencing of the *SUB* genes was performed by Microsynth (Balgach, Switzerland).

Standard PCR PCRs were performed in a PTC 200 thermal cycler (Biozym, Landgraaf, The Netherlands) using a Promega PCR Core System kit. Two hundred nanograms of genomic DNA or 10 ng of plasmidic DNA, 2 μl of deoxynucleotide mix (containing 10 mM of each dNTP), 1 μl of each of the sense and antisense oligonucleotides at a concentration of 42 μM, and 2 U of *Taq* DNA polymerase (Promega) were dissolved in a final volume of 50 μl of PCR buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100). The reaction mixtures were incubated for 5 min at 94°C, subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and finally incubated for 10 min at 72°C.

Reverse transcription PCR (RT-PCR) Total RNA was isolated from *M. canis* using the filamentous fungi protocol of a Qiagen RNeasy total RNA purification kit. RT-PCRs were performed using a One-step RT-PCR kit (Qiagen). Briefly, 1 μg of total RNA, 1 μl of oligonucleotides 1 and 2, 7 and 8, 9 and 10 for *SUB1*, *SUB2*, and *SUB3*, respectively (**Table I**), at a 42 μM concentration each, 2 μl of dNTPs at 10 mM each, 2 μl of One-step RT-PCR enzyme mix, and 1 U of *Pfu* DNA polymerase (Promega) were dissolved in a final volume of 50 μl RT-PCR buffer (12.5 mM $MgCl_2$, pH 8.7). The reaction mixtures were incubated at 50°C for 30 min, at 95°C for 15 min, and then subjected to 35 cycles of 1 min at 94°C, 1 min at 55°C, and 4 min at 68°C. RT-PCRs were completed by a final elongation step at 68°C for 10 min.

RT-nested PCR RT-nested PCRs were performed in order to assess the transcription of *SUB*s *in vivo*. Wood's light positive *M. canis*-infected hairs were plucked from infected guinea pigs on days 14 and 21 post-inoculation. Controls consisted of Wood's light negative noninfected hairs plucked from outside the scarification zone of the same animals, and from inside the scarification zone of noninfected guinea pigs. Approximately 20 hairs of each animal's sample were ground under liquid nitrogen using mortar and pestle, and total RNA was extracted using the filamentous fungi protocol of a Qiagen RNeasy total RNA purification kit. To avoid DNA contamination, RNA was treated with RNAse-free DNAse according to the manufacturer's instructions (Promega). For each reaction a control was performed omitting the RT step. RT-PCR was performed as described above except that template concentration was at least 10-fold lower and that the reaction mixture was subjected to 47 cycles of amplification. Primers 1–2, 5–6, 9–10 (**Table I**) were used to amplify the mRNA of *SUB1*, *SUB2*, and *SUB3*, respectively. Five microliters of RT-PCR products were then used as template in a nested PCR with the internal primers 3–4, 7–8, and 11–12 specific for *SUB1*, *SUB2*, and *SUB3*, respectively (**Table I**). The nested PCR procedures were otherwise identical to those described for standard PCR. The RT-nested PCR products were sequenced to definitively confirm the amplification of specific mRNAs.

RESULTS

Cloning of three genes encoding SUBs from *M. canis* Based on the N-terminal sequence homology between the *M. canis* 31.5 kDa keratinase isolated by Mignon *et al* (1998a) and *A. fumigatus* ALP (Jaton-Ogay *et al*, 1992), a first screening of the *M. canis* λEMBL3 genomic library was performed using the *ALP* gene as a probe and under low-stringency conditions of hybridization. Restriction enzyme analysis with *Eco*RI of the purified DNA of about 20 hybridizing bacteriophages revealed two differents groups of clones carrying similar, but not identical, DNA fragments. By southern analysis performed with the same probe, *SUB1* and *SUB2* were assigned to a 2.2 kb and a 3.5 kb *Eco*RI fragment, respectively. These genomic fragments were subcloned into pMTL21. Sequencing revealed two long open reading frames of 1566 bp and 1519 bp for *SUB1* and *SUB2*, respectively. The gene encoding the 31.5 kDa keratinase previously purified could not be isolated by this first screening. In order to clone this gene, a 840 bp PCR product corresponding to an internal fragment of the latter was amplified using degenerated oligonucleotide primers that were based on the N-terminal amino acid sequence of the mature protease (Mignon *et al*, 1998a) and on a highly conserved amino acid sequence in subtilisins (Rawlings and Barrett, 1994). Using this PCR product as a probe, 12 hybridizing bacteriophages were obtained, allowing identification of a 4 kb *Xho* I DNA fragment. Sequencing revealed an open reading frame of 1413 bp allowing identification of the N-terminal amino acid sequence of the 31.5 kDa protease, which was termed SUB3 (**Fig 1**). Sequencing

Table I Primers used for the isolation of *M. canis* IHEM 15221 SUBs cDNAs by RT-PCR and for the detection of the *in vivo* transcription of *M. canis* SUBs by RT-nested PCR

Genes	Primer number	Oligonucleotide primer (sequence)	Orientation	Encoded amino acid sequence	Template	PCR product length (with cloning sites)
SUB1	1	(CTTGTTC/TCGAGAAAAGAGG-CCACATTCTTTCTATGGGTC)	Sense[b]	(L)(V)(L)(E)(K)(R)GHILSMG	Total RNA	1419 bp (Xho I – Not I)
	2	(CTTGTTGC/GGCCGCGTTCC-AGAATCTGCCGAAGGTC)	Antisense[b]	TFGRFWN(A)(A)(A)(T)		
	3	(CCATCACTATCCACGGAG)	Sense[c]	ITIHG	cDNA	388 bp
	4	(CTGGCTAGCATGCTGGGC)	Antisense[c]	AQHASQ		
SUB2	5	(GTTGTTCTGCA/GTCGAGAT-TGCTCCACAGCCTGAG)	Sense[b]	(C)(S)(A)(V)EIAPQPE	Total RNA	1233 bp (Pst I – Not I)
	6	(GTTGTTGC/GGCCGCCTTAG-TGTTCTTGGGCAG)	Antisense[b]	LPKNTK(A)(A)(A)(T)		
	7	(CTCGCCTCTGCGCAGATC)	Sense[c]	LASAQI	cDNA	1010 bp
	8	(GGGCAGGTTCTCGAGGCT)	Antisense[c]	SLENLP		
SUB3	9	(GTTC/TCGAGAAAAGACGCG-CTTTCTTTCACAACCGT)	Sense[b]	(L)(E)(K)(R)RAFFHNR	Total RNA	1149 bp (Xho I – Not I)
	10	(GTAGC/GGCCGCCTATCTTC-CACTTCCGTTGTA)	Antisense[b]	YNGSGRStop		
	11	(ATGGCAACGGCCATGGCAC)	Sense[c]	GNGHG	cDNA	480 bp
	12	(AGGCCATGGAGGTTCCGG)	Antisense[c]	GTSMA		

[a]In parentheses are shown amino acids encoded by the restriction enzyme site sequences and amino acids encoded by nucleic sequences added to the extremities of primers.
[b]Primers used for RT-PCR.
[c]Internal primers used for nested PCR.

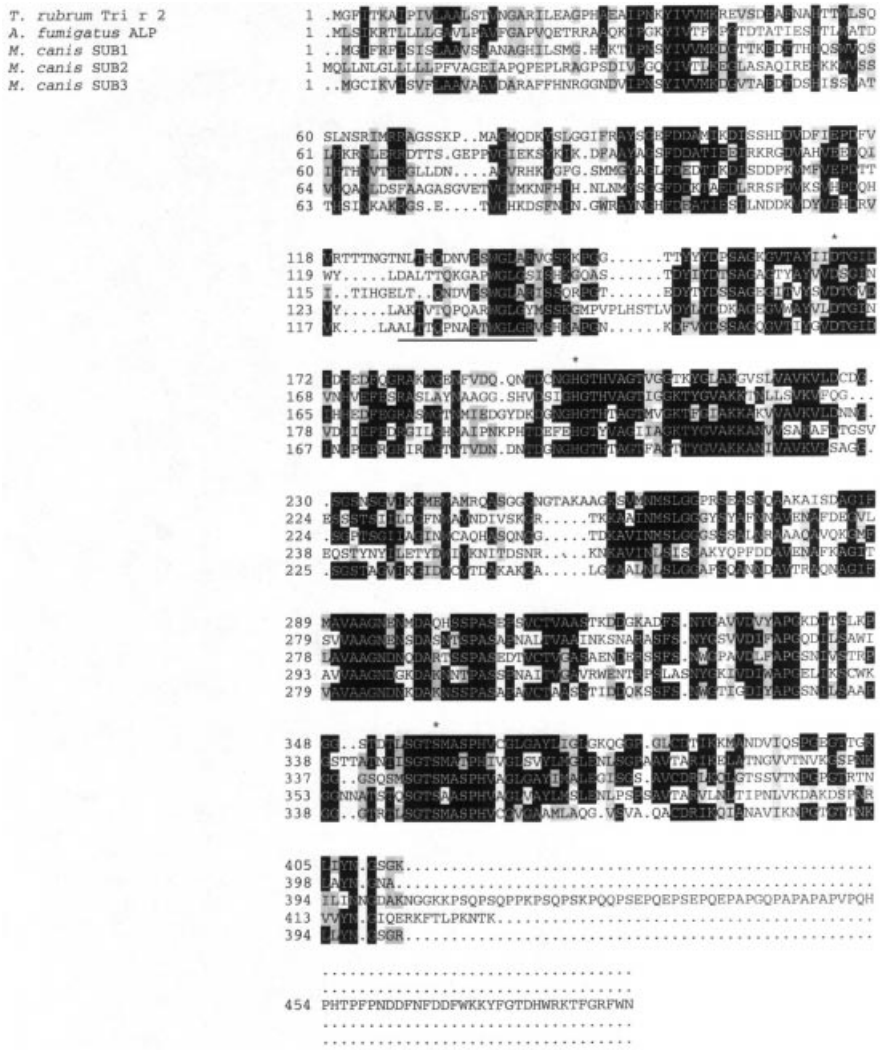

Figure 1. Alignment of deduced amino acid sequences showing large regions of identity between the *M. canis* IHEM 15221 SUB1, SUB2, and SUB3, *T. rubrum* Tri *r*2 (Woodfolk *et al*, 1998) and *A. fumigatus* ALP (Jaton-Ogay *et al*, 1992). Identical amino acids are black, homologous amino acids are gray shaded. The three amino acids of the catalytic triad of subtilisin proteases are labeled by an asterisk. N-terminal extremity of mature SUB3 (Mignon *et al*, 1998a) is underlined. The alignment was performed with the software Vector NTI and reformatted with Boxshade 3.31.

Table II Pairwise amino acid sequence comparisons between the SUBs from *M. canis* IHEM 15221 and from other filamentous fungi

Enzymes	% Identity[a]						
	M. canis SUB1	*M. canis* SUB2	*M. canis* SUB3	*T. rubrum* Tri r 2	*A. fumigatus* ALP	*A. flavus* serine protease	*A. nidulans* alkaline protease
M. canis SUB2	27.4						
M. canis SUB3	41.2	34.6					
T. rubrum Tri r 2	38.9	28.9	49.5				
A. fumigatus ALP	33.3	44	41.1	40.1			
A. flavus serine protease	34.5	43.3	41.4	38.6	77.2		
A. nidulans alkaline protease	34.3	43.8	42.5	41.7	78.2	74.5	
A. oryzae alkaline protease	34.3	43.6	39.7	39.3	81.9	76.4	74.5

[a]The percent identity values were obtained with the software Vector NTI (InforMax, Bethesda, MD).

Table III Molecular characteristics of the *M. canis* IHEM 15221 SUBs

	M. canis SUBs		
	SUB1	SUB2	SUB3
Preproprotein length (amino acids)	485	427	397
Signal sequence cleavage site position (residues number)[a]	19–20	17–18	19–20
Mature protein domain length (amino acids)	ND[b]	ND	281
Theoretical molecular mass of the preproprotein (kDa)[c]	51.3	46.1	40.8
Theoretical molecular mass of the polypeptide chain of the mature domain (kDa)[c]	ND	ND	28.2
Apparent molecular mass determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (kDa)[d]	ND	ND	31.5
Calculated[c]/measured[d] pI	ND	ND	9.2/11.8
Number of putative glycosylation sites	3	5	3

[a]Signal sequence cleavage site positions were predicted according to the method of von Heijne (1986).
[b]ND, not determined.
[c]The theoretical molecular masses and the pI were obtained with the software Vector NTI.
[d]The apparent molecular mass and pI of native SUB3 were described by Mignon *et al* (1998a). **Table 2**.

of RT-PCR products corresponding to the amplification of mRNAs of *SUB1*, *SUB2*, and *SUB3*, obtained from *M. canis* grown *in vitro*, confirmed previous genomic DNA sequencing. The amino acid sequences deduced from the three genes showed a quite high percentage of identity both between themselves and with SUBs from *T. rubrum* (Woodfolk *et al*, 1998), *A. fumigatus* (Jaton-Ogay *et al*, 1992), *Aspergillus oryzae* (Tatsumi *et al*, 1988), *Aspergillus nidulans* (Katz *et al*, 1994), and *Aspergillus flavus* (Ramesh *et al*, 1994) (**Table II**). Moreover, the three sequences displayed the catalytic triad (Asp/His/Ser) in the order common in all proteinases of the subtilisin family (**Fig 1**) and the subtilisin-characteristic motifs around these active site residues (Rawlings and Barrett, 1994). Deduced amino acid sequence analysis revealed that the N-terminal extremity of SUB3, previously determined for the native protein (Mignon *et al*, 1998a), was preceded by a preprosequence (**Fig 1**). Putative preprosequences and signal sequence cleavage sites were also found for SUB1 and SUB2 suggesting that the three proteases are synthesized as preproproteins (**Table III**). SUB1 C-terminal extremity showed a remarkable abundance in proline residues, in addition to several repeated short sequences (**Fig 1**). Molecular characteristics of SUBs are summarized in **Table III**.

Detection of the *in vivo* transcription of *SUB1*, *SUB2*, and *SUB3* in *M. canis*-infected guinea pig hair RT-nested PCR products, obtained from total RNA extracted from *M. canis*-infected hair of guinea pigs 14 and 21 d post-inoculation, were visualized on agarose gel as bands of approximately 380, 1000, and 480 bp corresponding to amplified fragments of *SUB1*, *SUB2*, and *SUB3* cDNAs, respectively (**Fig 2**). No signal was detected under the same conditions in control extracts. RT-PCR performed in the absence of reverse transcriptase did not show any *SUB* signals after the amplification procedure (data not shown). Moreover, the RT-nested PCR amplified fragments were sequenced, confirming the amplification of specific mRNAs.

DISCUSSION

We report here the cloning of the gene *SUB3* encoding the previously described *M. canis* 31.5 kDa keratinolytic subtilisin-like serine protease. This keratinase is of particular interest as it is the major polypeptide secreted by the fungus in a culture medium containing cat keratin as the sole nitrogen source and it is produced *in vivo* during skin infection (Mignon *et al*, 1998a). SUB3 expressed in *Pichia pastoris* (data not shown) displayed both apparent molecular weight and keratinolytic activity similar to those recorded with the native protein, confirming that *SUB3* is the gene encoding the 31.5 kDa *M. canis* keratinase. Two other genes, *SUB1* and *SUB2*, encoding homologous proteases were also cloned in this study. Homology between SUB1, SUB2, and SUB3 was revealed by the identification of the catalytic triad as well as conserved residues typical of serine proteases of the subtilisin family (Rawlings and Barrett, 1994) and by the high amino acid identity percentage. These results demonstrate that *M. canis* contains a family of SUBs. This could be important for a further investigation of *M. canis* infection pathogenesis, because proteases encoded by a gene family have been shown to be related to virulence in other fungal infections, such as secreted aspartyl proteinases (SAPs) of *Candida albicans* (Monod *et al*, 1994) and *Candida tropicalis* (Zaugg *et al*, 2001). Several authors (Monod *et al*, 1994; Zaugg *et al*, 2001) postulated that, in the establishment of an infection, the use of

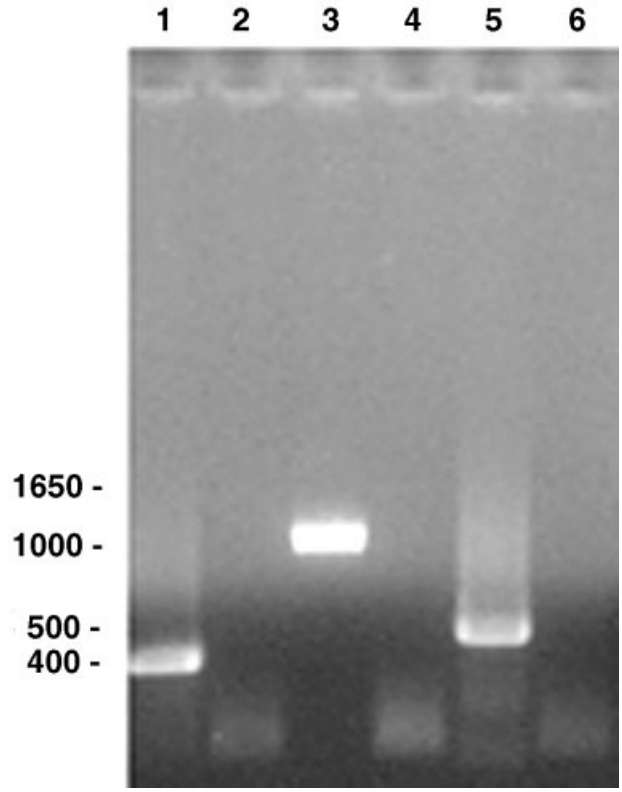

Figure 2. *Microsporum canis* IHEM 15221 *SUB* genes are transcribed *in vivo*. *Lanes 1*, *3*, *5*: RT-nested PCR products obtained from *M.* canis-infected guinea pig hairs, corresponding to internal cDNA fragments of *SUB*1, *SUB*2, and *SUB*3, respectively. *Lanes 2*, *4*, *6*: RT-nested PCR products obtained from noninfected guinea pig hairs. RT-nested PCR procedures are described in *Materials and Methods*. Ten microliter aliquots were electrophoresed in a 0.8% agarose gel and the bands were visualized by ethidium bromide staining. Molecular mass standards (in bp) are shown on the left.

combinations of different specific proteinases suitable to each particular condition can be required. Therefore, expansions of genes to form a gene family could reflect a selection during evolution allowing the organisms a better adaptation to the different conditions of their environment.

In order to investigate the potential role of the different SUBs in the pathogenesis of *M. canis* dermatophytosis, their *in vivo* expression was evaluated in *M. canis*-infected guinea pigs. Previously, SUB3 was shown to be expressed *in vivo*, in the hair of both naturally infected cats and experimentally infected guinea pigs (Mignon *et al*, 1998b; 1999a), using immunohistochemistry. Despite the fact that this technique, in contrast to PCR, is suitable for protein detection, it was not used in this study. Indeed, given the important homology between SUBs, possible antibody–antigen cross-reactions could not be excluded. Consequently, RT-nested PCRs using specific primers for each *SUB* were performed using total RNA extracted from *M. canis*-infected hair. Another advantage of RT-nested PCR is that it alleviates the lack of sensitivity that the *in vivo* detection of gene products by classical RT-PCR can suffer from (Naglik *et al*, 1999; Okeke *et al*, 2001). Under the experimental conditions used, *SUB1, SUB2*, and *SUB3* were shown to be transcribed during *M. canis* experimental dermatophytosis. Further experiments are necessary to demonstrate that SUB1 and SUB2 are finally translated *in vivo*, as was shown for SUB3, and to determine whether the proteases are expressed by the mycelium invading the keratinized structures, as suggested by Mignon *et al* (1998a), or by the arthroconidia, which are considered as the most infective form of the fungus (Zurita and Hay, 1987).

The deduced amino acid sequence of the *M. canis*-secreted SUBs also showed high percentages of identity with secreted proteases from the filamentous fungi *T. rubrum* (Woodfolk *et al*, 1998), *A. fumigatus* (Jaton-Ogay *et al*, 1992), *A. oryzae* (Tatsumi *et al* (1988), *A. flavus* (Ramesh *et al* (1994), and *A. nidulans* (Katz *et al* (1994). Other sequence homologies between *M. canis* and *Aspergillus* spp. proteases have already been reported. Indeed, the N-terminal extremity sequence of the 43.5 kDa *M. canis* keratinolytic metalloprotease (Brouta *et al*, 2001) disclosed marked similarities with those of metalloproteases from *A. fumigatus* (MEP) (Monod *et al*, 1993) and *A. oryzae* (Doumas *et al*, 1999). Moreover, the recent molecular characterization of the 43.5 kDa *M. canis* keratinase gene (*MEP*) showed that it was homologous to genes encoding the latter metalloproteases (Brouta *et al*, unpublished results). These results strengthen the hypothesis (Brouta *et al*, 2001) according to which *M. canis* and *Aspergillus* spp., both members of the *Onygenales* family, would share fundamental similarities in their proteolytic system, even though they produce proteinases with different specificities related to the substrates they hydrolyze and to the tissues they can invade.

In experimentally infected guinea pigs, a SUB3-containing exoantigen was previously shown to elicit strong delayed type hypersensitivity skin reactions whereas weak reactions were observed using purified SUB3 only (Mignon *et al*, 1999a). Moreover, specific IgG antiexoantigen were detected both in these guinea pigs (Mignon *et al*, 1999a) and in naturally infected cats (Mignon *et al*, 1999b). In contrast, no anti-SUB3 specific antibody could be detected. These results suggest that major *M. canis* antigens remain to be identified. In this context, the isolation of two additional protease genes (*SUB1* and *SUB2*), whose *in vivo* transcription has been demonstrated in this study, must be taken into account. Interestingly, the carboxy-terminal extremity of SUB1 showed a remarkable abundance of proline residues in addition to several small repeated segments. The biologic significance of this part of the molecule is unclear. Several parasite extracellular antigens contain proline-rich domains (Dalrymple *et al*, 1993; Accosta-Serrano *et al*, 2001), which could be implicated in epitope variability and immune evasion (Van Regenmortel *et al*, 1997). Nevertheless, these antigens are encoded by gene families, which does not seem to be the case for SUB1. The role of the latter protease in the host immunomodulation, which can be observed in dermatophytosis (Hay, 1997), remains therefore to be established.

This is the first report describing the isolation of a gene family encoding potential virulence-related factors in dermatophytes. The multiplicity of *M. canis* SUBs and their *in vivo* transcription in guinea pig infected hair in addition to the keratinolytic activity of at least SUB3 make it necessary to investigate further their potential implication in the fungal infection. In this context, site-directed mutagenesis experiments will be performed in the near future.

We thank Barbara Léchenne, Jacques Detry, Humbert Gianfreda, and Nicole Gérardin for excellent technical assistance. This work was supported by grant 3.4534.1 from Fonds de la Recherche Scientifique Médicale (FRSM) and by grant 3200-063697.0 from Fonds National Suisse pour la Recherche Scientifique. Frédéric Descamps and Frédéric Brouta are the recipients of a studentship of F.R.I.A. (Fonds pour la Formation à la Recherche dans l'1ndustrie et dans l'Agriculture, rue d'Egmont 5, 1000 Bruxelles).

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